Detection of *Escherichia coli* O157:H7 by Multiplex PCR

In order to develop a PCR assay for *Escherichia coli* O157:H7, a portion of the 60-MDa plasmid harbored by enterohemorrhagic *E. coli* (EHEC) was sequenced and PCR primers were designed. A multiplex PCR method was then designed by employing primers specific for the EHEC *eaeA* gene, conserved sequences of Shiga-like toxins I (SLT-I) and II (SLT-II), and the 60-MDa plasmid. PCR products of 1,087 bp (*eaeA*), 227 and/or 224 bp (SLT-I and/or SLT-II), and 166 bp (plasmid) were successfully amplified simultaneously in a single reaction. The multiplex PCR method can be used to specifically identify EHEC of serogroup O157.

Escherichia coli O157:H7 has emerged as a foodborne pathogen of considerable public health importance. Virulence factors include the production of one or more bacteriophage-encoded Shiga-like toxins (SLT) (13, 22), also called verotoxins, and the ability to intimately adhere to the intestinal mucosa by an attaching and effacing mechanism (23). Virtually all *E. coli* O157:H7 strains harbor a large plasmid (ca. 60 MDa) (5). It is currently unknown if the plasmid plays a role in the organism's virulence.

The enterohemorrhagic *E. coli* (EHEC) *eaeA* gene has been cloned and sequenced, and it has been suggested that the protein product may be necessary for attaching and effacing adhesion (25). Louie et al. (16) determined that the EHEC *eaeA* gene product was a 97-kDa outer membrane protein which they called intimin$_{O157}$. The enteropathogenic *E. coli* *eaeA* gene which encodes a 94-kDa product and the EHEC *eaeA* gene share 97% homology at the 5′ ends but are only 59% homologous over the last 800 bp at the 3′-terminal regions (16). The genes which encode SLT-I and SLT-II have also been cloned and sequenced (10). Immunological and DNA-based methods have been developed for the detection of SLT-producing *E. coli* (1, 6, 9, 17) and for EHEC possessing the *eaeA* gene (7) and the large plasmid (14). Detection of 60-MDa plasmid sequences is currently limited to probes without amplification, since sequence data which would allow design of primers are not yet known. Cebula et al. (3) recently described a multiplex PCR assay for *E. coli* O157:H7 in which simultaneous amplification of SLT sequences and of the *uidA* (gene which encodes β-glucuronidase) allele in O157:H7 strains was achieved. In this study, we report on the use of a multiplex PCR assay for simultaneous amplification of SLT-I and/or -II, EHEC *eaeA*, and plasmid DNA sequences. The multiplex PCR described has the potential to be used as a sensitive, specific, and rapid method for clinical diagnosis of disease caused by SLT-producing *E. coli* O157 and may find application for direct PCR of enrichment cultures (2 to 4 h) of fecal specimens. Direct multiplex PCR of fecal cultures is more rapid than conventional culturing on MacConkey sorbitol agar, and with PCR there is less possibility of missing positive cases of disease caused by SLT-producing *E. coli* O157.

Sequencing of plasmid DNA and PCR. Plasmid pCVD419 (pBR325 containing the 3.4-kb fragment of the 60-MDa plasmid of *E. coli* O157:H7 strain (933) was kindly provided by James Nataro (Center for Vaccine Development, University of Maryland School of Medicine, Baltimore) (14). pCVD419 was digested with *Hin*dIII, and the isolated 3.4-kb fragment was then digested with *Eco*RI, yielding fragments of approximately 1.4 and 1.9 kb in size. The 1.4-kb fragment was subcloned into M13 mp19 with an M13 cloning kit (Boehringer Mannheim Corporation, Indianapolis, Ind.) according to the manufacturer's instructions. A portion of the 1.4-kb fragment was then sequenced with the Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio). Two PCR primers (Table 1) were designed to specifically amplify a 166-bp amplicon within the 60-MDa plasmid sequence. A gene bank search using FASTA (18) revealed a 63.4% homology between the sequenced fragment and a 435-bp fragment of the chromosomal *E. coli hlyA* gene (4). Our results are in agreement with Schmidt et al. (20) who recently reported that the large plasmid (ca. 60 MDa) of *E. coli* O157:H7 EDL933 is associated with hemolytic activity and that the gene encoding this hemolysin shared ca. 70% homology with the *hlyA* gene present in other pathogenic *E. coli* serotypes.

For PCR amplification of the plasmid fragment, a bacterial colony was transferred from nutrient agar (Difco Laboratories, Detroit, Mich.) to 200 μl of a solution consisting of 0.5% Triton X-100, 20 mM Tris (pH 8.0), and 2 mM EDTA, and the bacterial suspensions were heated at 100°C for 10 min. The PCR reaction (total volume of 100 μl) consisted of 5 to 10 μl of the crude cell lysates, 1.5 mM $MgCl_2$, 20 mM Tris (pH 8.0), 50 mM KCl, 0.001% gelatin, 200 μM each of the four deoxynucleoside triphosphates, 2.5 U of *Taq* DNA polymerase (Gibco/BRL, Gaithersburg, Md.), and 50 pmol of each primer. PCRs were performed in a thermal cycler (MJ Research, Inc., Watertown, Mass.) using the following cycling conditions: an initial denaturation at 94°C for 5 min and 35 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C. The amplified product was visualized following ethidium bromide staining of agarose (1.6%) gels. To confirm the identity of the 166-bp PCR product, amplified DNA was analyzed following gel electrophoresis by Southern blotting (19) using a 3′-end-labeled (digoxigenin-11-ddUTP) internal oligonucleotide probe (PS28) (Genius 5 kit, Boehringer Mannheim location), 5′-CCGT ATCTTATAATAAGACGGATGTTGG-3′. A 166-bp hybridization signal was visible with all of the strains in which the amplification product was detectable on agarose gels (data not shown).

To determine the sensitivity of the PCR, serial 10-fold dilutions in 1% peptone were prepared from a 4-h culture of *E. coli*

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TABLE 1. Primers used in multiplex PCR for detection of SLT-producing *E. coli* O157

Primer	Sequence (5′ to 3′)	Target(s)	Reference
MFS1F	ACGATGTGGTTTATTCTGGA	60-MDa plasmid	This study
MFS1R	CTTCACGTCACCATACATAT		This study
MK1	TTTACGATAGACTTCTCGAC	SLT-I and SLT-II	11
MK2	CACATATAAATTATTTCGCTC		11
AE 19	CAGGTCGTCGTGTCTGCTAAA	*eaeA*	7
AE 20	TCAGCGTGGTTGGATCAACCT		7

O157:H7 strain B1409 grown in brain heart infusion broth (Difco). Following PCR amplification, the products were subjected to agarose (1.6%) gel electrophoresis. A 166-bp PCR product was generated with as little as 1.2 CFU (Fig. 1A) (sensitivity ranged from 3 to 1.2 CFU). The PCR products were transferred to nylon membranes by Southern blotting and hybridized with the internal probe 3′ tailed with digoxigenin-11-dUTP/dATP prepared with a Genius 6 kit (Boehringer Mannheim), and bands were detected by using a Genius 3 kit (Boehringer Mannheim) according to the manufacturer's instructions. Sensitivity of the PCR by colorimetric detection was 0.12 CFU (Fig. 1B) (sensitivity ranged from 3 to 0.12 CFU). A sensitivity of less than 1 CFU may have been due to the presence of free plasmid in the growth medium released from ruptured bacteria.

Primers MFS1F and MFS1R were tested in the PCR with crude cell lysates from 153 bacterial strains (Table 2). PCR results of all of the *E. coli* O157:H7, SLT$^+$ O157:NM, and O157:H$^-$ strains tested showed a 166-bp amplification product on agarose gels. PCR of several other *E. coli* serotypes such as O26:H11 and O103:H2 also resulted in a 166-bp product. All of the non-O157 strains which were PCR positive for the 60-MDa plasmid also possessed SLT DNA sequences (Table 2).

Multiplex PCR. Primers used for amplification of SLT-I, SLT-II, and *eaeA* sequences are listed in Table 1. Primers used for amplification of *eaeA* were reported by Gannon et al. (7) to be highly specific for *E. coli* serogroup O157. The three sets of primers, MFS1F and MFS1R, MK1 and MK2 which amplify fragments of 227 and 224 bp of the SLT-I and SLT-II genes, respectively, and AE 19 and AE 20 which amplify a 1,087-bp fragment of the EHEC *eaeA* gene, were added in equal concentrations to the PCR. When the cycling conditions employed for amplification of the plasmid fragment were used, only the plasmid and *eaeA* products were visualized by gel electrophoresis. Modifications in the cycling protocol resulted in successful amplification of plasmid, SLT, and *eaeA* products in one PCR. The multiplex PCR cycling conditions were as follows: an initial denaturation at 94°C for 5 min followed by 35 cycles, with 1 cycle consisting of 1 min at 94°C, 3 min at 48°C, and 4 min at 72°C. However, using a Perkin Elmer 480 thermal cycler (Perkin Elmer, Norwalk, Conn.), amplification of the three products also occurred with the following cycling conditions: 3 min at 94°C for 1 cycle followed by 35 cycles, with 1 cycle consisting of 45 s at 94°C, 30 s at 55°C, and 90 s at 72°C, with a final chain elongation at 72°C for 10 min. Although the 166-bp product is generated with as little as 1.2 CFU, in the multiplex PCR the detection limit of the *eaeA* gene product is ca. 100 CFU and the detection limit of the SLT gene is ca. 1,000 CFU. The multiplex PCR conditions used are apparently more favorable for amplification of plasmid and *eaeA* sequences and less favorable for SLT sequences. Also the plasmid may be present in multiple copies, whereas *eaeA* and SLT genes may be present as single copies. Multiplex PCR conditions are currently being optimized in order to increase sensitivities for the SLT and *eaeA* targets. The specificity of the multiplex PCR for *E. coli* O157:H7 was evaluated with 61 bacterial strains including 16 *E. coli* O157:H7 strains, 8 *E. coli* O157 (NM and H$^-$) strains, *E. coli* strains of other serotypes, *Shewanella putrefaciens*, and *Pseudomonas aeruginosa* (Table 2). Since the multiplex PCR can potentially be applied to test both food samples and fecal specimens, organisms such as *S. putrefaciens* which can be found in food and water and *P. aeruginosa* which can be associated with human infections and can also be isolated from skin and feces of normal humans were tested. Amplification products of the expected sizes for plasmid and SLT and *eaeA* genes were obtained with all *E. coli* strains of serotypes O157:H7, O157:NM, and O157:H$^-$ except for one nontoxigenic O157:NM strain which was negative for all three products. It is not surprising that there were no amplification products with the nontoxigenic O157:NM strain, since Schmidt et al. (21) found that SLT-negative *E. coli* O157 did not hybridize with gene probe CVD 419 (14). Therefore, these strains appear to lack the EHEC 60-MDa plasmid but possess an *eaeA* gene similar to that of enteropathogenic *E. coli*. By using a primer pair specific for the EHEC *eaeA* gene, an amplification product was obtained only with SLT-positive *E. coli* O157. Toxigenic *E. coli* O157:NM strains are frequently associated with cases of hemorrhagic colitis and hemolytic uremic syndrome (2, 8), therefore, the described PCR assay which specifically detects and identifies *E. coli* O157:H7 and nonmotile toxigenic O157 strains will be an important tool for diagnosis of these organisms.

The multiplex PCR assay described here gives a positive signal with all three primer pairs only for toxigenic O157 strains while other *E. coli* strains are negative for at least one primer set. *E. coli* O91:H21 and O125:NM isolates and one O26:H11 and one O55:H7 isolate possessed SLT sequences but were negative in the PCR with plasmid primers. Both plasmid sequence and SLT gene sequences were amplified in several *E. coli* serotypes including O111:NM (1 of 4), O26:H11 (4 of 5), O145:NM (1 of 2), O5:NM (1 of 3), O103:H2 (3 of 3), O45:H2 (1 of 1), and O22:H8 (1 of 1). In none of these strains was the *eaeA* sequence amplified. Multiplex PCR results

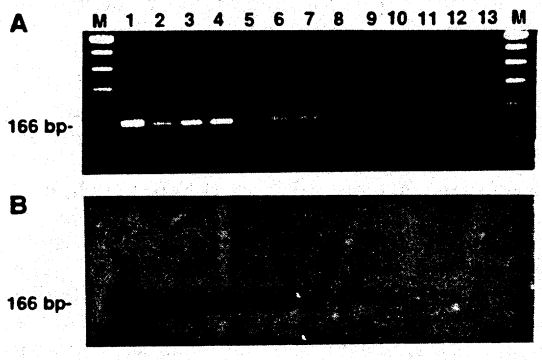

FIG. 1. Sensitivity of PCR in detecting the plasmid sequence by using serial 10-fold dilutions of *E. coli* O157:H7 strain B1409. The 166-bp amplification products were analyzed by electrophoresis through a 1.6% agarose gel followed by ethidium bromide staining (A) and by Southern hybridization with a digoxigenin-labeled internal probe (B) (colorimetric detection). Lane 1, 1.2×10^7 CFU used in PCR; 2, 1.2×10^6 CFU; 3, 1.2×10^5 CFU; 4, 1.2×10^4 CFU; 5, 1.2×10^3 CFU; 6, 1.2×10^2 CFU; 7, 12 CFU; 8, 1.2 CFU; 9, 0.12 CFU; 10, 0.012 CFU; 11, 0.0012 CFU; 12, 0.00012 CFU; 13, H_2O negative control; M, molecular size markers (100-bp DNA ladder; Gibco/BRL). Southern hybridization was more sensitive for detection of the plasmid amplicon (B) than agarose gel electrophoresis (A). The expected mobility of the 166-bp fragment is shown.

TABLE 2. PCR results of bacterial strains tested in this study

Bacterial strain	No. of strains	No. of strains showing positive PCR result/no. of strains tested			
		PCR using MFS1F and MFS1R primers	Multiplex PCR		
			Plasmid	SLTs	*eaeA*
E. coli					
O157:H7	32	32/32	12/12	12/12	12/12
O157:H7 P^{-}[c]	4	0/4	0/4	4/4	4/4
O157:NM (SLT^{+})[b]	6	6/6	6/6	6/6	6/6
O157:NM (SLT^{-})[c]	1	0/1	0/1	0/1	0/1
O157:H^{-}	1	1/1	1/1	1/1	1/1
O26:H11	5	4/5	4/5	5/5	0/5
O111:NM	4	1/4	1/4	1/4	0/4
O145:NM	2	1/2	1/2	1/2	0/2
O5:NM	3	1/3	1/3	1/3	0/3
O4:NM	2	0/2	0/2	0/2	0/2
O125:NM	1	0/1	0/1	1/1	0/1
O103:H2	3	3/3	3/3	3/3	0/3
O45:H2	1	1/1	1/1	1/1	0/1
O22:H8	1	1/1	1/1	1/1	0/1
O91:H21	1	0/1	0/1	1/1	0/1
O113:H21	1	0/1	0/1	0/1	0/1
O55:H7	3	0/3	0/3	1/3	3/3
O78:H12	2	0/1	0/1	0/1	0/1
O78:H11	1	0/1	0/1	0/1	0/1
O29:NM	1	0/1	0/1	0/1	0/1
O25:NM	1	0/1	0/1	0/1	0/1
K-12 C600	1	0/1	0/1	0/1	0/1
HB101(pCVD419) (14)	1	1/1	NT[d]	NT	NT
HS	1	0/1	NT	NT	NT
JM109	1	0/1	NT	NT	NT
JM103	1	0/1	NT	NT	NT
V517	1	0/1	NT	NT	NT
J53 (R16)	1	0/1	NT	NT	NT
ML35	1	0/1	NT	NT	NT
B ATCC 11303	1	0/1	NT	NT	NT
Shewanella putrefaciens	1	0/1	0/1	0/1	0/1
Pseudomonas aeruginosa	5	0/5	0/1	0/1	0/1
Pseudomonas fluorescens	3	0/3	0/3	0/3	0/3
Shigella dysenteriae	1	0/1	NT	NT	NT
Shigella flexneri	2	0/2	NT	NT	NT
Shigella sonnei	1	0/1	NT	NT	NT
Salmonella typhimurium	5	0/5	NT	NT	NT
Salmonella enteriditis	4	0/4	NT	NT	NT
Salmonella arizonae	1	0/1	NT	NT	NT
Salmonella anatum	1	0/1	NT	NT	NT
Salmonella seftenberg	1	0/1	NT	NT	NT
Salmonella dublin	2	0/2	NT	NT	NT
Salmonella poona	1	0/1	NT	NT	NT
Aeromonas hydrophila	2	0/2	NT	NT	NT
Staphylococcus aureus	4	0/4	NT	NT	NT
Vibrio parahemolyticus	1	0/1	NT	NT	NT
Yersinia enterocolitica	4	0/4	NT	NT	NT
Serratia marcescens	1	0/1	NT	NT	NT
Serratia liquefaciens	1	0/1	NT	NT	NT
Rhodococcus equis	1	0/1	NT	NT	NT
Listeria monocytogenes	18	0/18	NT	NT	NT
Listeria innocua	1	0/1	NT	NT	NT
Carnobacterium piscicola	1	0/1	NT	NT	NT
Streptococcus faecium	2	0/2	NT	NT	NT
Bacillus subtilis	2	0/2	NT	NT	NT
Bacillus cereus	1	0/1	NT	NT	NT
Bacillus thuringiensis	1	0/1	NT	NT	NT

[a] 60-MDa plasmid-cured strains (5).
[b] SLT producers.
[c] Negative for SLT production.
[d] Not tested by multiplex PCR.

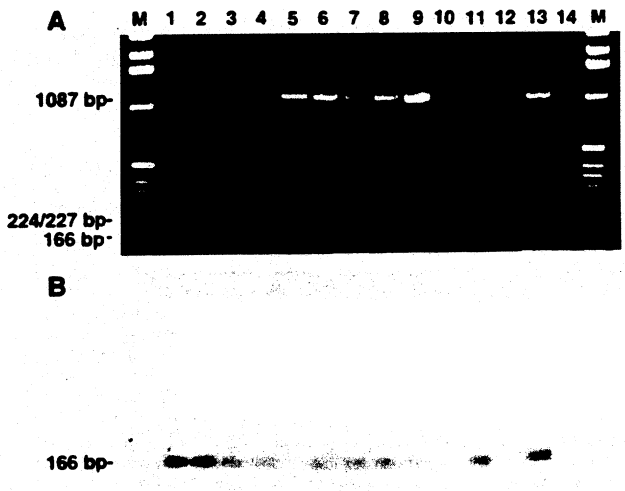

FIG. 2. Amplification products obtained by multiplex PCR of selected bacterial strains analyzed by agarose (1.6%) gel electrophoresis (A) and by Southern blot hybridization (B) with a digoxigenin-labeled DNA plasmid internal probe (chemiluminescent detection). Lane 1, *E. coli* O42:H2 88.0501; 2, *E. coli* O5:NM 85.0587; 3, *E. coli* O103:H2 93.0626; 4, *E. coli* O111:NM 88.0015; 5, *E. coli* O157:H7 933P^{-}; 6, *E. coli* O157:H7 933; 7, *E. coli* O157:H7 A9124-1; 8, *E. coli* O157:H7 B1409; 9, *E. coli* O157:NM MF13180; 10, *E. coli* O29:NM ATCC 43892; 11, *E. coli* O26:H11 3359-70; 12, *E. coli* O111:NM 90.2152; 13, *E. coli* O157:H^{-}; 14, H_2O negative control; M, molecular size markers (1-kb ladder, Gibco/BRL). The expected mobilities of the 1087-, 224 and/or 227-, and 166-bp fragments are shown.

showed an amplification product of the expected size (1,087 bp) with the three *E. coli* O55:H7 strains tested by using primers AE 19 and AE 20 which are specific for the EHEC *eaeA* gene. These results are not surprising, since Whittam et al. (24) showed by multilocus enzyme electrophoresis that the O157:H7 clone was most closely related to *E. coli* O55:H7, which has been recognized as a cause of diarrheal disease. They suggested that these two serotypes may have very similar *eae* genes since both form attaching and effacing lesions on intestinal epithelial cells. Louie et al. (15) also recently reported that serotypes O157:H7 and O55:H7 strains have almost identical nucleotide and amino acid sequences in regions where the *eaeA* gene and protein product of enteropathogenic *E. coli* and EHEC differ. Examples of multiplex PCR results on several bacterial strains are shown in Fig. 2A. Following Southern blotting of the gel and hybridization with the internal probe (PS28), a hybridization signal was visible only in strains in which the 166-bp fragment was amplified (Fig. 2B). The probe did not hybridize with the SLT or *eaeA* products.

SLT-producing *E. coli* serotypes such as O26:H11 and O111:NM have been associated with hemorrhagic colitis and hemolytic uremic syndrome (2, 13). However, the value of screening clinical specimens or food samples for the presence of SLT or SLT gene sequences is presently controversial, since other *E. coli* serotypes produce SLT and not all may be clinically significant in humans. Furthermore, Karch et al. (12) reported that clinical *E. coli* isolates may lose SLT genes upon subcultivation which would cause false-negative results. Screening for the EHEC *eaeA* gene may give positive results with *E. coli* other than O157:H7 (e.g., O55:H7). Although virtually all EHEC O157 strains possess the 60-MDa plasmid, several other SLT-producing *E. coli* serotypes also harbor this plasmid. Therefore, a detection method relying only on sequences specific for this plasmid will not allow specific detection of EHEC O157 strains. The above-mentioned multiplex PCR should prove to be a very useful method, since virulence

gene (SLT-I and -II and *eaeA*) and 60-MDa plasmid sequences are targeted simultaneously, therefore allowing specific detection of EHEC O157 strains.

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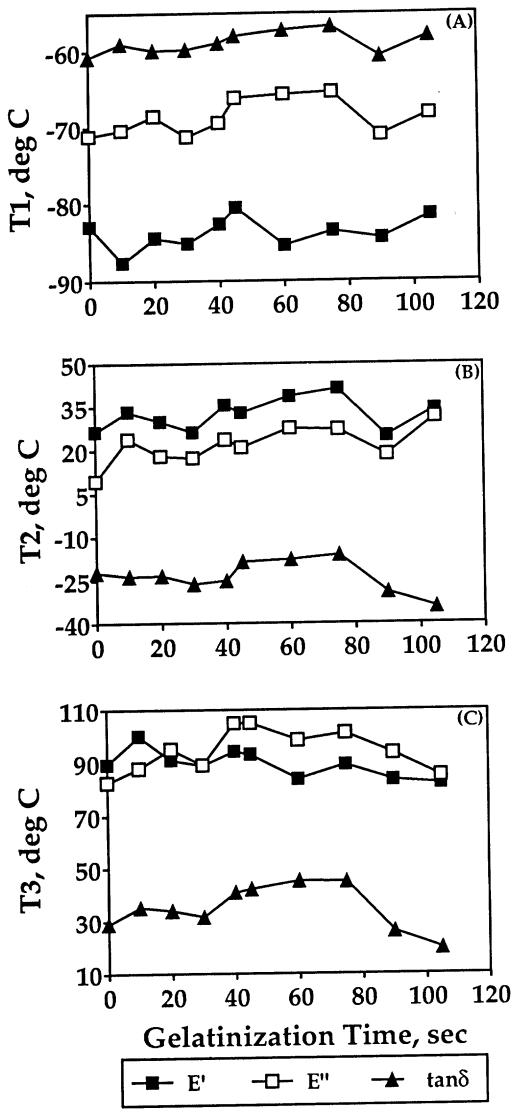

Figure 4 Effect of gelatinization time in glycerol/water on temperature of thermal transitions in E′, E″, and tan δ plots. (A) T_1, (B) T_2, (C) T_3.

or minimum. For T_2, E′ showed a moderate increase with increasing gelatinization time, although the value dropped sharply for 105 s. E″ showed no strong trend with gelatinization time, but did exhibit a minimum at 40 s. There was no trend for the loss tangent value. At T_3, no trend was seen for E′, whereas a moderate downward trend with increasing gelatinization time was seen for E″. There also appeared to be a minimum for E″ at 40 s. A slight downward trend was seen with the loss tangent. The modulus trends are shown in Figure 5.

A comparison of the values for T_1, T_2, and T_3 between the samples where the starch was gelatinized in the presence of glycerol and those where it was gelatinized in its absence showed that the temperatures at which the transitions occurred were typically 10°C lower for the films made from starch gelatinized in the presence of glycerol. At the same time, the values for the storage modulus and loss modulus at these transitions did not differ significantly between the two methods of gelatinization.

Scanning Electron Microscopy

Fractured faces of both types of films displayed three general types of structural organization that were linked to gelatinization times. The first type included films made from mixtures gelatinized for 0 to 20 s.

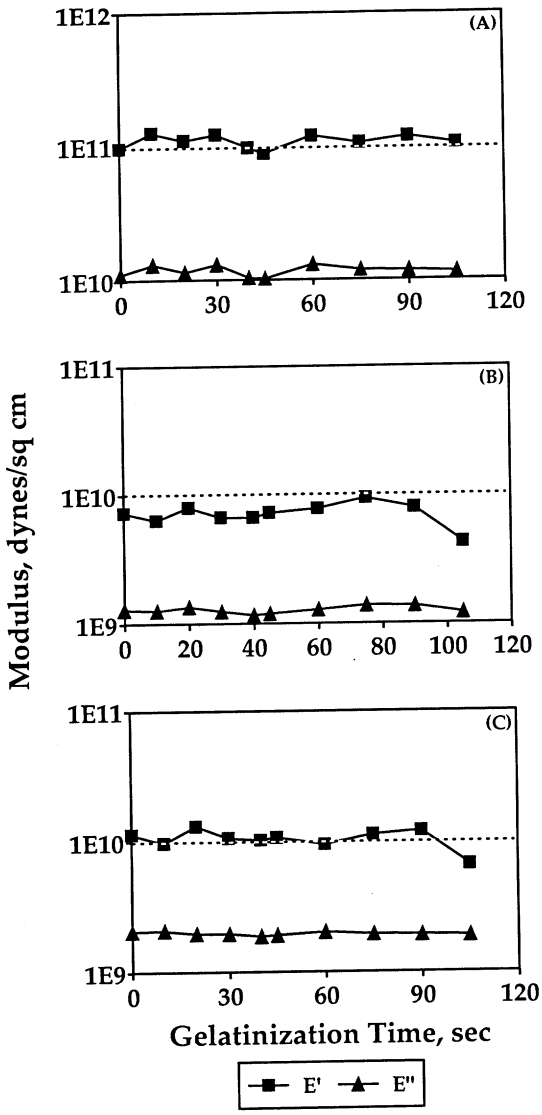

Figure 5 Effect of gelatinization time in glycerol/water on storage modulus (E′) and loss modulus (E″). (A) T_1, (B) T_2, (C) T_3.

These contained fractured and nonfractured starch granules ranging from 5–10 μm in diameter, which were uniformly distributed and embedded within a smooth, continuous pectin matrix [Fig. 6(a) and (d)]. The second type included films made from mixtures gelatinized for 30 to 60 s. In these, starch granules or recognizable remnants were rarely found. Instead, the films were composed of a homogeneous granular matrix that often split unevenly along the planes of fracture [Fig. 6(b) and 6(e). The third type of organization, present in films made from mixtures gelatinized for 75 to 105 s, was characterized by a uniform and close-packed distribution of 1 μm diameter particles embedded in a homogeneous smooth matrix [Fig. 6(c) and (f)].

DISCUSSION

The disappearance of the starch granules with increasing gelatinization time was an expected occurrence as the increasing gelatinization times result in higher temperatures being obtained (10). The nature of the microwave gelatinization process is such that the sample sees a continually increasing temperature as the exposure to the microwave radiation continues. Thus, longer gelatinization times lead to both higher exposure temperatures, and longer exposure to the elevated temperatures. As a consequence, the gelatinization time at which the starch granules disappear is a result of both kinetic and thermodynamic factors. Thirty sec was the shortest gelatinization time in which there was any indication that the starch slurry had been heated above room temperature.

Both sets of samples showed almost exactly the same trend for the disappearance of the starch granules with time. Thus, it would appear that the presence of the glycerol had little or no effect on the solubilization of the granules at the micrometer level of magnification.

For the samples using starch gelatinized in pure water, the minimum observed in the transition temperature/gelatinization time plots for T_1, T_2, and T_3 occurred just beyond this point at gelatinization times of 40–45 s. This would seem to indicate that the starch granules were essentially completely disrupted after 30 s of gelatinization, but that slightly more time was necessary to more completely disrupt the intermolecular structure on a smaller scale and allow the starch molecules to mix more intimately in with the pectin. The samples made using starch gelatinized in the presence of glycerol did not show the distinct minimum in the transition temperatures found with the other films, however. The decrease seen in the transition temperatures of the films made with the starch gelatinized in the presence of glycerol relative to those observed where the starch had been gelatinized in pure water may indicate an enhanced degree of mixing between the two biopolymers.

The presence or absence of glycerol had only a small effect on film moduli values or starch granule size. In general, the storage and loss modulus both declined moderately with increasing gelatinization time both with and without glycerol in the gelatinization bomb. However, these declines were on the order of 20% or less.

Apparently, changes in the starch microstructure had a relatively small effect on the overall modulus behavior of the films, except perhaps in compositions made using gelatinization times of 40–45 s. Possibly the starch is acting more as a filler than as a secondary polymeric component. The somewhat higher modulus values observed at the lower gelatinization times could be a result of the starch granules acting as particulate reinforcers of the pectin matrix. The volume fraction of the 1 μm particles seen at the higher gelatinization times may not be large enough to cause the same matrix reinforcement that occurred at larger granule size (i.e., at gelatinization times of less than 30 s).

Some degree of gelatinization seems to be necessary to obtain a visually acceptable film. Films made using the ungelatinized starch showed a layered surface pattern that suggested there was separation of the starch granules from the casting solution during the drying step. The layered pattern was not visible in micrographs of films with starch gelatinized for 10 s, the shortest gelatinization time employed.

CONCLUSIONS

The microstructure of films made from blends of high methoxy pectin and gelatinized high amylose starch was significantly affected by the heating time used to gelatinize the starch. Starch granules disappeared at heating times between 20 and 30 s, and much smaller starch particles seemed to reappear at gelatinization times of 75 s or more. These changes in structure resulted in only modest changes in the mechanical properties of the films as measured by storage modulus and loss modulus.

The temperatures of three selected transitions on the modulus curves were relatively unaffected by gelatinization time, except at times of 40–45 s in the absence of glycerol in the gelatinization solutions

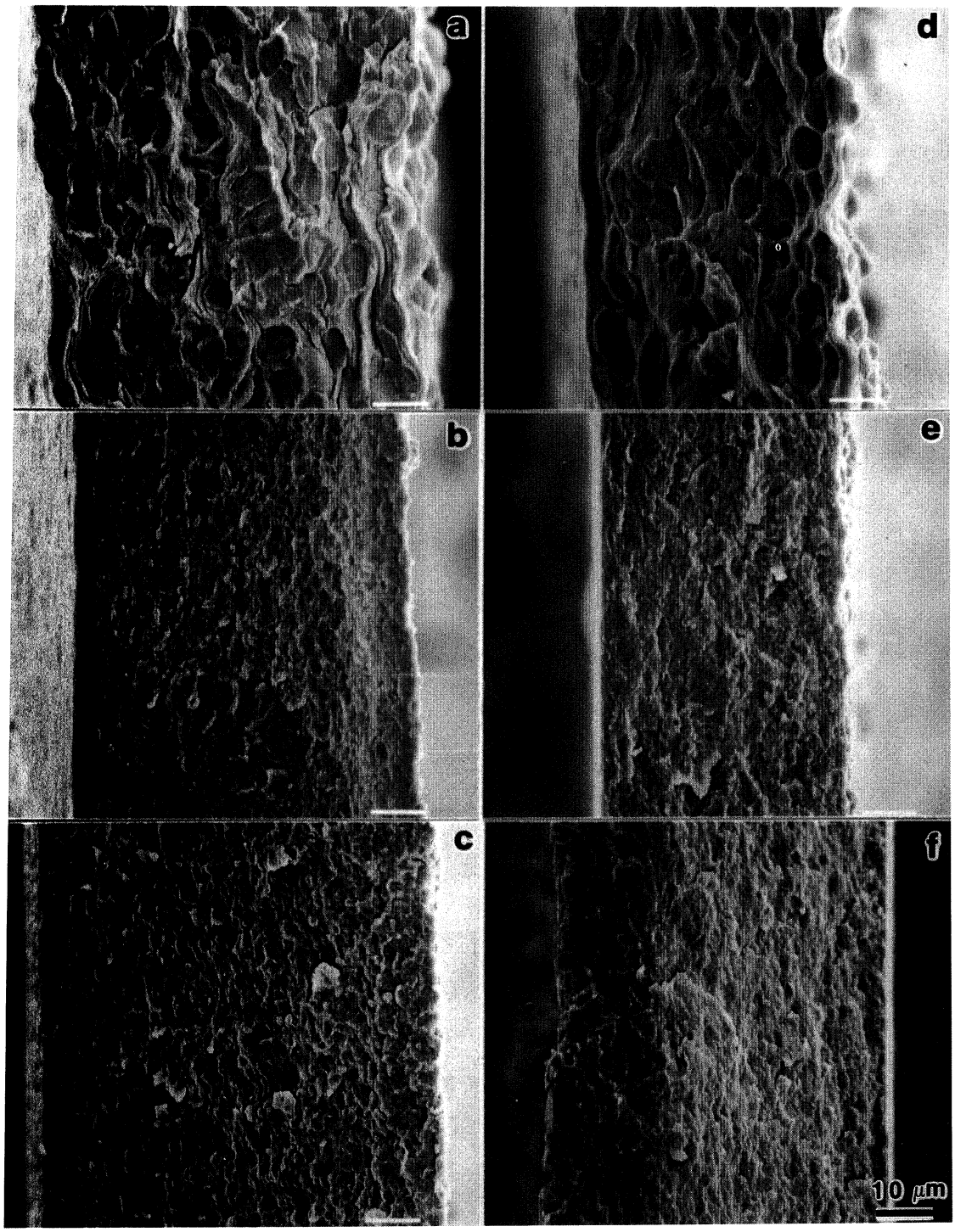

Figure 6 Electron micrographs of film cross-sections. Starch gelatinized in water; (A) 20 s gelatinization time, (B) 45 s gelatinization time, (C) 90 s gelatinization time. Starch gelatinized in glycerol/water; (D) 20 s gelatinization time, (E) 45 s gelatinization time, (F) 90 s gelatinization time.

where the transitions were lowered by 10–25°C. This minimum also tended to show up for the two moduli as well under both types of gelatinization conditions, although it was not usually as pronounced. The transitions were all lowered by about 10°C when the gelatinization solutions contained the glycerol to be used for the plasticizer, possibly indicating an enhanced degree of mixing of the starch and pectin.

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